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De novo sequencing of two novel peptides homologous to calcitonin-like peptides, from skin secretion of the Chinese Frog, *Odorrana schmackeri*[☆]

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ABSTRACT

An MS/MS based analytical strategy was followed to solve the complete sequence of two new peptides from frog (*Odorrana schmackeri*) skin secretion. This involved reduction and alkylation with two different alkylating agents followed by high resolution tandem mass spectrometry. *De novo* sequencing was achieved by complementary CID and ETD fragmentations of full-length peptides and of selected tryptic fragments. Heavy and light isotope dimethyl labeling assisted with annotation of sequence ion series. The identified primary structures are GCD[I/L]STCATHN[I/L]VNE[I/L]NKFDKSKPSSGGVGPESF-NH₂ and SCNLSTCATHNLVNELNKFDDKSKPSSGGVGPESF-NH₂, i.e. two carboxyamidated 34 residue peptides with an aminoterminal intramolecular ring structure formed by a disulfide bridge between Cys₂ and Cys₇. Edman degradation analysis of the second peptide positively confirmed the exact sequence, resolving I/L discriminations. Both peptide sequences are novel and share homology with calcitonin, calcitonin gene related peptide (CGRP) and adrenomedullin from other vertebrates. Detailed sequence analysis as well as the 34 residue length of both *O. schmackeri* peptides, suggest they do not fully qualify as either calcitonins (32 residues) or CGRPs (37 amino acids) and may justify their classification in a novel peptide family within the calcitonin gene related peptide superfamily. Smooth muscle contractility assays with synthetic replicas of the S–S linked peptides on rat tail artery, uterus, bladder and ileum did not reveal myotropic activity.

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1. Introduction

Many anuran amphibians have developed a defensive mechanism in which specialized dorsal granular skin glands secrete biologically active compounds. Part of these bioactive compounds are peptides that have a wide variety in bioactivity and they are considered interesting for their pharmacological activity [1].

[☆] With our deepest respect, we want to dedicate this paper to the memory of Juan Pablo Albar, who generously synthesized both newly discovered peptides reported here, and, therefore, posthumously features as co-author.

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Amongst the skin secretory peptides, broad-spectrum antimicrobial peptides are predominantly present [2–4]. Besides, several frog skin secretions contain peptides that show remarkable similarity with regulatory peptides found in the vertebrate system [1,5]. Already 30 years ago, Vittorio Ersparmer, one of the pioneers in amphibian skin peptides, introduced the ‘brain-gut-skin’ triangle, postulating that every peptide present in frog skin has its equivalent in the mammalian brain or gut [6,7]. Since then, the discovery of many frog skin peptides strengthened this statement. Therefore, studying frog skin peptides may not only provide useful information on the biochemistry in frog defense system, but it may also provide leads for the discovery of new regulatory peptides in higher vertebrates, including man. Amphibian skin secretory

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peptides are often post-translationally modified and due to the lack of complete genome information, this makes them difficult to sequence using the proteomics/peptidomics mass spectrometry based approaches [8,9]. Previously, we reported a method to selectively screen animal venoms for disulfide bond containing peptides using two dimensional peptide mass displays [10]. This type of post-translational modification is found in many bioactive peptides, such as, among others, vasopressin, oxytocin, insulin, to which it renders a typical tertiary ring structure to maintain biological activity and conformational stability. Skin secretory peptides from *Odorrana* species often have a single disulfide bond located in the C-terminal 5–8 amino acids, the so called 'Rana-box'. The global analysis showed that the skin secretion of *Odorrana schmackeri* contains a multitude of single disulfide bond containing peptides. From this initial screening of disulfide bonds containing peptides secreted by *O. schmackeri* defense glands we discovered two yet unknown peptides. Their characteristic shift in mass and chromatographic retention time after reduction and alkylation, was indicative of an intramolecular disulfide bridge. The location of the disulfide bond on the two novel peptides however was found to be N-terminal, which is rather unusual for *Odorrana* peptides, and this prompted us to do full structure elucidation of these two peptides as presented in this work.

2. Materials and methods

2.1. Skin secretion sample

Lyophilized sample of *O. schmackeri* defensive skin secretion (frog 'venom') was used in this study. The material was non-invasively collected by gentle electrical stimulation, essentially as described before [11].

2.2. Initial LC MS/MS analysis: ETD and CID fragmentation

Three equivalent batches (0.5 mg) of crude 'venom' of *O. schmackeri* were prepared for LC MS/MS analysis (ThermoFisher Scientific LTQ-Orbitrap XLTM). All were reduced with 5 mM dithiothreitol (DTT). One batch was alkylated with 10 mM iodoacetamide (IAM) and the second with 10 mM bromoethylamine (BrEA). The third aliquot was not alkylated.

Prior to chromatography the resolubilized material was centrifuged at 13,000 rpm to remove insoluble material. The 3 samples were separated by nano HPLC using an Agilent 1200 series HPLC system (Agilent Technologies, CA, USA). Of each sample the equivalent of 100 ng lyophilized skin secretion was injected. Peptides were trapped and desalted on a trapping column (100 μ m ID \times 2 cm length), packed with C4 particles (Reprosil C4, 5 μ m particles, Dr. Maisch, Ammerbuch-Entringen, Germany) for 10 min at 5 μ L/min in 100% Solvent A (MilliQ water with 0.6% acetic acid). Peptide separation on an analytical column (50 μ m ID \times 25 cm length, packed with Reprosil C4, 5 μ m particles) was achieved in a 100 min gradient from 0% to 80% solvent B (80% ACN, 20% MilliQ water and 0.6% acetic acid). During LC MS/MS analysis, the two most intense ions from the survey scan were automatically selected for fragmentation by CID and ETD. FT MS and FT MS/MS spectra were recorded respectively at 60K and 30K resolution, in positive ionization mode and as profile data. For ETD and CID, a normalized collision energy of 35 eV was applied, with an activation Q of 0.25 ms each and activation time of 30 ms for CID and 50 ms for ETD; supplementary activation was enabled for ETD.

2.3. Semi-preparative HPLC peptide enrichment/purification

A 4 mg aliquot of *O. schmackeri* skin secretion was fractionated by reverse phase HPLC (Waters 2695 AllianceTM, Manchester, UK)

on a C8 column (Zorbax-XD, 5 μ m particles, 4.6 mm ID \times 15 cm length, Agilent Technologies) employing a 40 min linear gradient (at 1 mL/min flow rate) from 10% to 80% methanol with 0.05% trifluoroacetic acid (TFA). Of the column effluent 10% was directed to the ESI-source of a Q-TOF MS/MS system (Waters QTOF PremierTM, Manchester, UK) to acquire an *m/z* based chromatogram, while the 90% portion of the effluent was collected in 2 min fractions. The peptides targeted in this study (designated OsCTLPS, see below) eluted at 30 and 32 min (resp. fractions #15, OsCTLP-1 and #16, OsCTLP-2). These were used in the follow-up experiments for sequence analysis.

2.4. Trypsin digestion

Of each fraction (#15 and #16) 100 μ L (5%) were vacuum dried and reconstituted in 50 μ L of 25 mM ammonium bicarbonate (ABC) buffer, pH 8.0. Disulfide bonds were reduced with 2 mM DTT and free thiol groups were alkylated with 4 mM IAM. Half of each fraction was digested by adding 1 μ g/mL trypsin in solution (overnight at room temperature). Tryptic digests were separated by nanoflow HPLC on a 50 μ m ID \times 20 cm length C18 column (Reprosil C18-AQ, Dr. Maisch, 5 μ m particles), using a gradient from 0 to 50% ACN in 45 min (Solvent A: MilliQ water with 0.6% acetic acid, Solvent B: 80% ACN, 20% MilliQ water and 0.6% acetic acid). NanoLC analysis of both treated fractions was by online linear ion trap-orbitrap MS/MS (ThermoFisher Scientific LTQ-Orbitrap VelosTM, Bremen, Germany). FT MS and MS/MS spectra were acquired at 7.5 K and 100 K resolution, respectively. The *m/z* corresponding to the targeted OsCTLPS (with modified cysteines) and their tryptic fragments were selected for collision induced dissociation, using an isolation window of 3 Da, a normalized collision energy of 35, activation Q of 0.25 and an activation time of 30 ms. The mass spectrometer was operated in positive ion mode, data were acquired in the mass range between 400 and 2000 *m/z*, and recorded as profile data.

2.5. Carboxypeptidase assay

As one of the peptides (OsCTLP-2) did not yield unequivocal tandem MS sequence data, particularly at the C-terminal half of the peptide, 5% (100 μ L) of the respective fraction #16 was vacuum dried and reconstituted in 5 mM tri-sodium citrate (TSC) pH 6.0. Cystines were reduced by 5 mM *tris*(2-carboxyethyl) phosphine (TCEP) prior to the enzyme assay.

Carboxypeptidase Y (CPY, Sigma–Aldrich) was added at a concentration of 0.26 mg/mL and incubated at 37 °C (water bath). Aliquots of 1 μ L were taken at 0, 2, 5, 15, 30, 60, 100, 125, 155, 180 and 200 min after the reaction started and the exoprotease reaction was quenched immediately by mixing with an equal volume of α -cyano-4-hydrocinnamic acid (matrix) dissolved in 49.5% acetonitrile in water containing 1% TFA. The resulting sample was directly spotted onto a MALDI target plate. MS analysis was carried out on a MALDI-QTOF-MS (Waters Q-TOF PremierTM).

2.6. Dimethyl labeling

Dimethyl labeling with formaldehyde and deuterated formaldehyde was performed essentially as described by Boersema et al. [12]. Part of the trypsinized fraction #16 was vacuum dried and reconstituted in triethylammonium bicarbonate buffer and split in two. Either 4 μ L conventional or 4 μ L deuterated formaldehyde (4% solution) were added to the peptide solution together with 4 μ L of 0.6 M cyanoborohydrate solution. This mixture was left to react for 1 h at room temperature, after which the labeling was stopped by addition of 16 μ L 1% ammonia solution. Heavy and light labeled peptides were mixed and analyzed by nanoLC MS/MS on

our linear ion trap-orbitrap hybrid instrument (LTQ-Orbitrap Velos™, ThermoFisher). Dimethyl labelled peptides were separated by nanoflow HPLC on a C18 column (Reprosil C18 AQ, 5 μ m particles, Dr. Maisch; 50 μ m ID \times 20 cm length) using a gradient from 0 to 50% ACN in 45 min (solvent A: MilliQ water with 0.6% acetic acid, Solvent B: 80% ACN, 20% MilliQ water and 0.6% acetic acid). CID peptide fragmentation was achieved using a normalized collision energy of 45, resolution 30 K, activation Q of 0.25, activation time 30 ms and 2.5 Da isolation width.

2.7. De novo sequence analysis and homology searches

The obtained mass spectra were deconvoluted (Thermo Xcalibur™ Xtract) and protein *de novo* sequencing was done manually. The complete sequence was submitted for a BLAST search against the NCBI non-redundant and SwissProt/UniProt databases.

2.8. Edman degradation of OsCTLP-2

OsCTLP-2 was purified from 10% of the respective HPLC fraction #16 (200 μ L dried). The dried material was resuspended in 50 μ L 50 mM ABC buffer, pH 8.0, reduced with 5 mM DTT for 30 min at RT and subsequently alkylated with 10 mM IAM for 45 min in the dark at RT. Next, the modified peptides were fractionated by HPLC (Waters 2695 Alliance, Manchester, UK) using a C4 column (Reprosil C4, Dr. Maisch; 5 μ m particles; 2.0 mm ID \times 15 cm length) at a flow rate of 0.25 mL/min. Solvent A was 0.005% TFA in milliQ water and solvent B was 80% acetonitrile, 0.004% TFA. The first 5 min of the separation were run isocratically at 100% A, followed by a linear increase of 1% B/min for 50 min. Elution was

monitored at 215 nm, one minute fractions were collected. The peptide presence was confirmed by direct infusion of 10% of the fraction into the source of an LTQ-Orbitrap Velos™. Under these conditions, OsCTLP-2 was found to elute in subfraction #33 (32 min). Following, purified OsCTLP-2 was cleaved with 1 μ g trypsin in 100 mM TEAB buffer for 1.5 h at room temperature, and rechromatographed under identical conditions (same C4 column, gradient and solvents). The N-terminal tryptic fragment of OsCTLP-2 eluted at 31 min and its C-terminal part at 16 min. The respective fractions OS#16-#33#32 and OS#16-#33#17, were dried and analyzed by automated Edman degradation on a pulsed liquid-phase sequencer, (Procise 492 cLC, Life Technologies, Grand Island, N.Y., USA), as previously described [13].

2.9. Peptide Synthesis

For precise bioactivity studies, both peptides were synthesized by Fmoc chemistry on an automated multiple peptide synthesizer (AMS 422, ABIMED Analysen-Technik GmbH, Langenfeld, Germany). The peptide integrity was verified by high resolution (orbitrap) MS analysis. The disulfide bond formation was promoted by addition of 10% of DMSO according to the method of Tam et al. [14], and the resulting oxidized peptides were purified by HPLC. Comparison of analytical LC MS/MS characteristics with the native peptides authenticated the identity of the synthetic and native peptides.

2.10. Bioactivity screening

As a first test for bioactivity, we selected various (rat) smooth muscle preparations, on which many frog myoactive peptides have

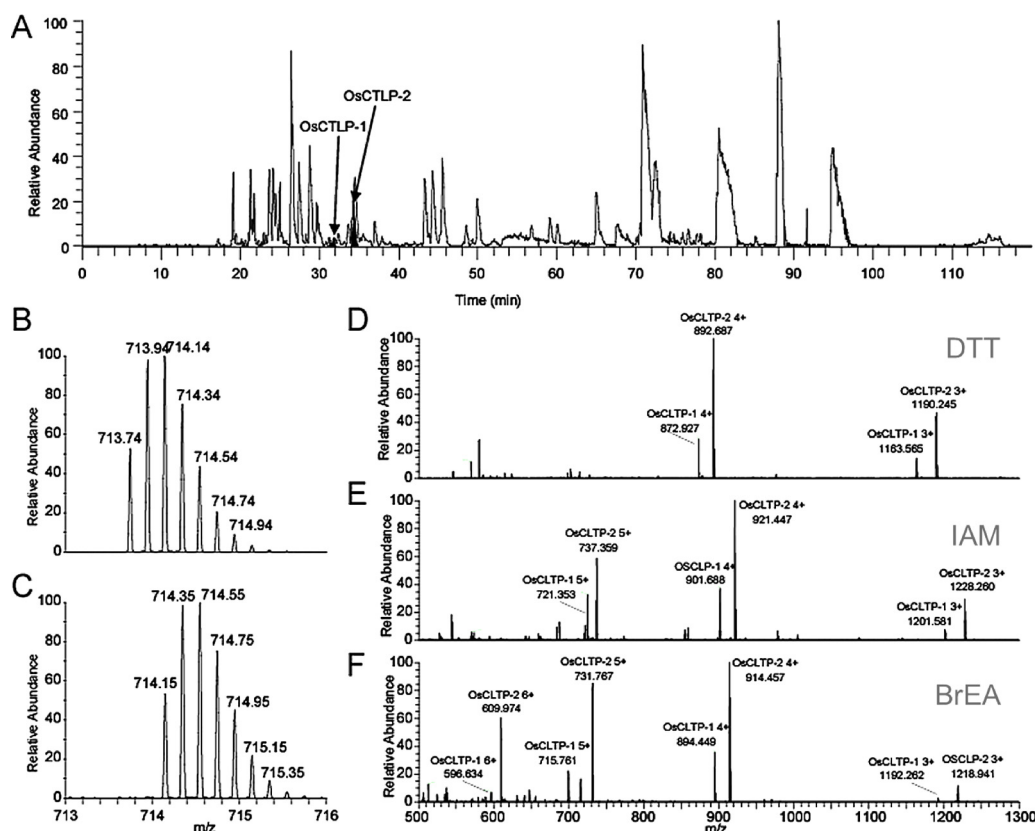


Fig. 1. (A) Base peak chromatogram of LC-MS analysis of untreated crude *Odorrana schmackeri* skin secretion. Elution position of both OsCTLP-1 and -2 are indicated with arrows. (B) Expanded part of spectrum of $[M+5H]^{5+}$ of unreduced form of OsCTLP-2. (C) Expanded part of spectrum of $[M+5H]^{5+}$ of DTT reduced form of OsCTLP-2. Full range spectrum showing charge state distribution of OsCTLP-1 and 2 in (D) DTT reduced form, (E) IAM alkylated form and (F) BrEA alkylated form.

shown pronounced effects. For this, smooth muscle preparations of rat tail artery, uterus, urinary bladder as well as ileum were done according to UK animal experimentation guidelines and ethics as described [15]. Both synthetic peptides (with their disulfide bridge properly formed) were tested at concentrations up to 10^{-6} M.

3. Results

Skin secretion peptides of *O. schmackeri* were separated by nano RP-HPLC and analyzed by high resolution orbitrap MS analysis. Fig. 1A shows the base peak intensity chromatogram, in which two peptides eluted at 34.3 min and 36.5 min with monoisotopic masses of 3484.639 and 3563.684 Da, respectively. CID fragmentation analysis of both native peptides showed a relatively poor degree of fragmentation (data not shown) and upon reduction with dithiothreitol, the mass of both peptides increased with 2.014 Da, as shown for one of the peptides in Fig. 1B. This mass increase is indicative for the presence of a single internal disulfide bond. Analysis of the fragmentation spectra of the reduced peptides showed that the location of this intermolecular disulfide bond is N-terminal (data not shown), which is unusual for skin peptides of *Odorrana* species. The partially elucidated primary structure showed a high degree of homology with calcitonin and this prompted us to do an in-depth analysis to obtain the full amino acid sequence. Here, we report the strategy that was used to *de novo* sequence both peptides using a combination of analytics involving different mass spectrometrical and biochemical tools. Because of the partial structural homology with members of the Calcitonin/CGRP/adrenomedullin superfamily of peptides, we designated the new peptides OsCTLP, for “*Odorrana schmackeri* calcitonin-like peptide”. We numbered them OsCTLP-1 and OsCTLP-2, reflecting their RP-HPLC elution order. Cysteine residues were reduced and alkylated with bromoethylamine (BrEA) or iodoacetamide (IAM) to avoid re-oxidation of the S–S bridges. It was observed that this treatment resulted in a shift of the

respective peptide ion distributions to higher charge states. This effect was most pronounced with BrEA as shown in Fig. 1C, and this made both peptides very suitable for ETD fragmentation analysis. Both peptides were subjected to targeted CID and ETD fragmentation analysis and by manual interpretation of spectra of various charge state precursor ions, both peptides could be sequenced almost completely. Both peptides are 34 residues in length and for OsCTLP-1 nearly 100% and for OsCTLP-2 ~75% of the amino acids could be proposed from this initial CID/ETD analysis.

The near complete sequence of OsCTLP-1 reads: GCD[L/I]STCATHN[L/I]VNE[L/I]NKFDKSKPSSGGVG[PE/EP]SP-NH₂ (Fig. 2), with only the Leu and Ile residues at positions 4, 12 and 16 remaining ambiguous, as well as the exact order of residues 3 and 4 from the C-terminus (ProGlu or GluPro). *De novo* sequencing OsCTLP-2 was more arduous, with more ambiguities to be elucidated due to interruptions of sequence ion series, particularly at the N-terminus and in the C-terminal half of the peptide: [SC/CS]N[L/I]STCATHN[L/I]VNE[L/I]NKFDKSK[PS/SP]SG[CV/VG]GPESF[-NH₂]. CID and ETD spectra of the peptide modified with IAM (Fig. 3) and BrEA (Fig. 4), besides the unidentified Leu and Ile residues, could not distinguish the exact order of several couples of residues, including aminoterminal residues 1 and 2, residues 24 and 25, residues 28 and 29.

As both OsCTLPs contained Lys residues halfway along the sequence, we decided to use trypsin to yield smaller peptides for more efficient CID fragmentation. Eventually, this confirmed the N-terminus of OsCTLP-1 (GCD[L/I]STCATHN[L/I]VNE[L/I]NK) and of OsCTLP-2 ([SC/CS]N[L/I]STCATHN[L/I]VNE[L/I]NK) as initially sequenced (Fig. 4). More importantly it enabled to fully *de novo* sequence the C-terminus of OsCTLP-1 (SKPSSGGVGPESP-NH₂) with the PE/EP ambiguity in OsCTLP-1 solved. By selecting the exact masses of the peptides to be fragmented, it was confirmed that both peptides were carboxyamidated. OsCTLP-1 was only detected in the amidated form (Fig. 5A), whereas a small proportion of OsCTLP-2 was detectable as not amidated (Fig. 5B).

DTT reduced form of OsCTLP-2. Full range spectrum showing charge state distribution of OsCTLP-1 and 2 in (D) DTT reduced form, (E) IAM alkylated form and (F) BrEA alkylated form.

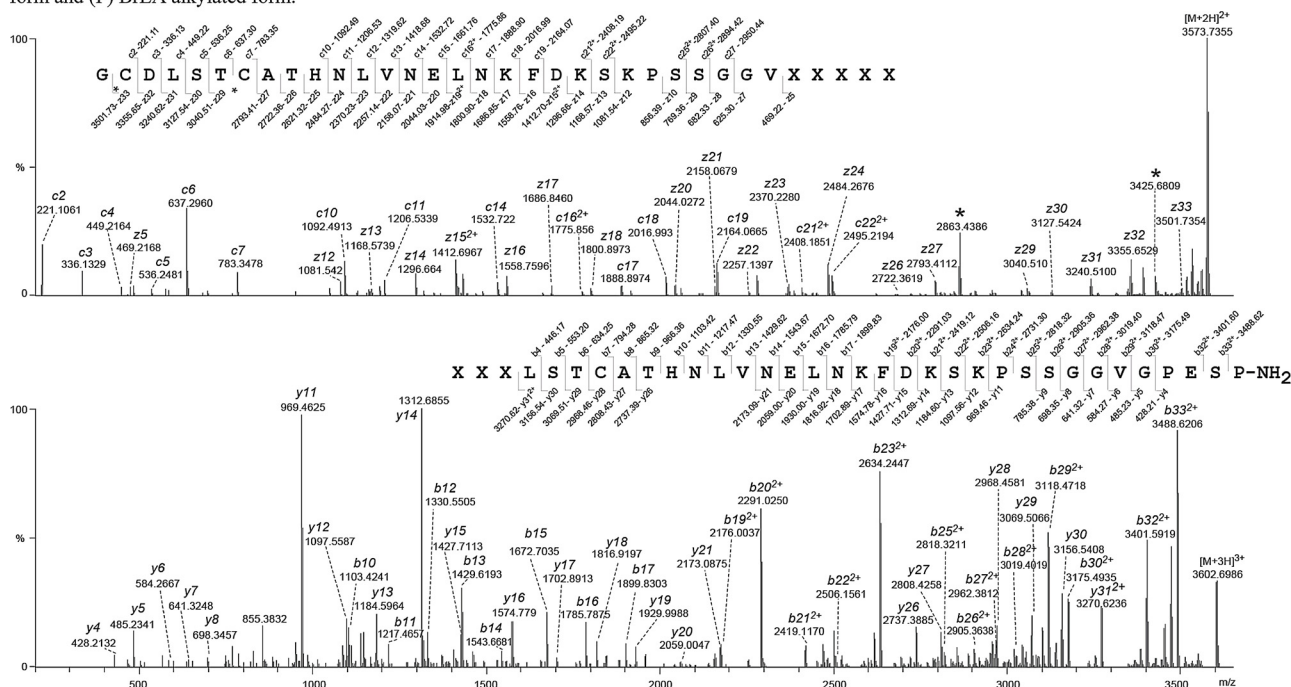


Fig. 2. Deconvoluted ETD (top, cysteines modified with BrEA) and CID (bottom, cysteines modified with IAM) spectra of OsCTLP-1. Ions *de novo* sequenced are assigned in spectra and labeled on peptide sequence above spectrum. Ions detected below 5% are not annotated, but are shown on sequence.

*Ions yielding specific side chain loss of modified cysteine (–76.022).

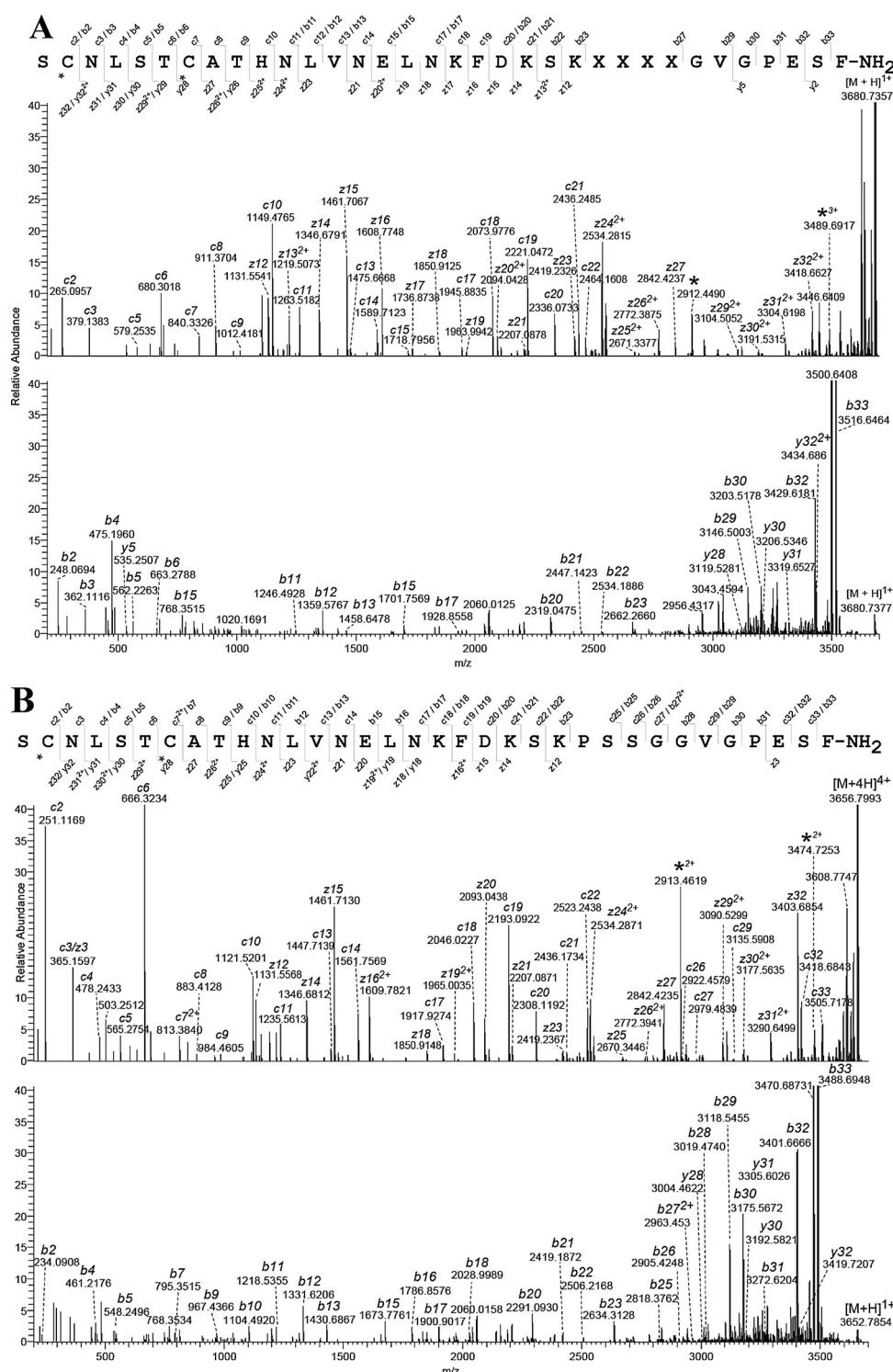


Fig. 3. Deconvoluted ETD (top) and CID (bottom) spectra of OsCTLP-2 with cysteines modified with IAM (A) and BrEA (B). Ions *de novo* sequenced are assigned in spectra and labeled on peptide sequence. Ions detected below 1 % are not annotated in spectra, but are shown on sequence.

*Ions from specific side chain loss of modified cysteines (–90.001 from carbamidomethyl cysteines (IAM) and –76.022 from ethylamine cysteines (BrEA)).

Still some sequence uncertainties remained in OsCTLP-2, even after tryptic fragment MS/MS: the exact order of the first two N-terminal residues as well as the amino acids between the 5th and 11th residue of the OsCTLP-2C-terminal tryptic fragment. MS analysis after carboxypeptidase Y treatment (see Materials and Methods, section 5) was attempted to resolve these issues. Exoprotease hydrolysis for 100 min showed OsCTLP-2 in most of its truncated forms (Fig. 6), allowing confirmation of the sequence

until the 10th position from the C-terminus as X_nSSGGVGPESF-NH₂). Still, the 11th residue from the carboxyl end of the peptide remained obscure (Fig. 6). Additional clues to clarify the amino acid order in the C-terminal fragment of OsCTLP-2 came from labeling with heavy (deuterated) and light dimethyl. This treatment yields a peptide mass increase of 32 or 28 Da, respectively, per free amine present in the sequence (amino-terminus and ε-amine of Lys side chain). In the non-amidated OsCTLP-2, y-ion peaks of both heavy

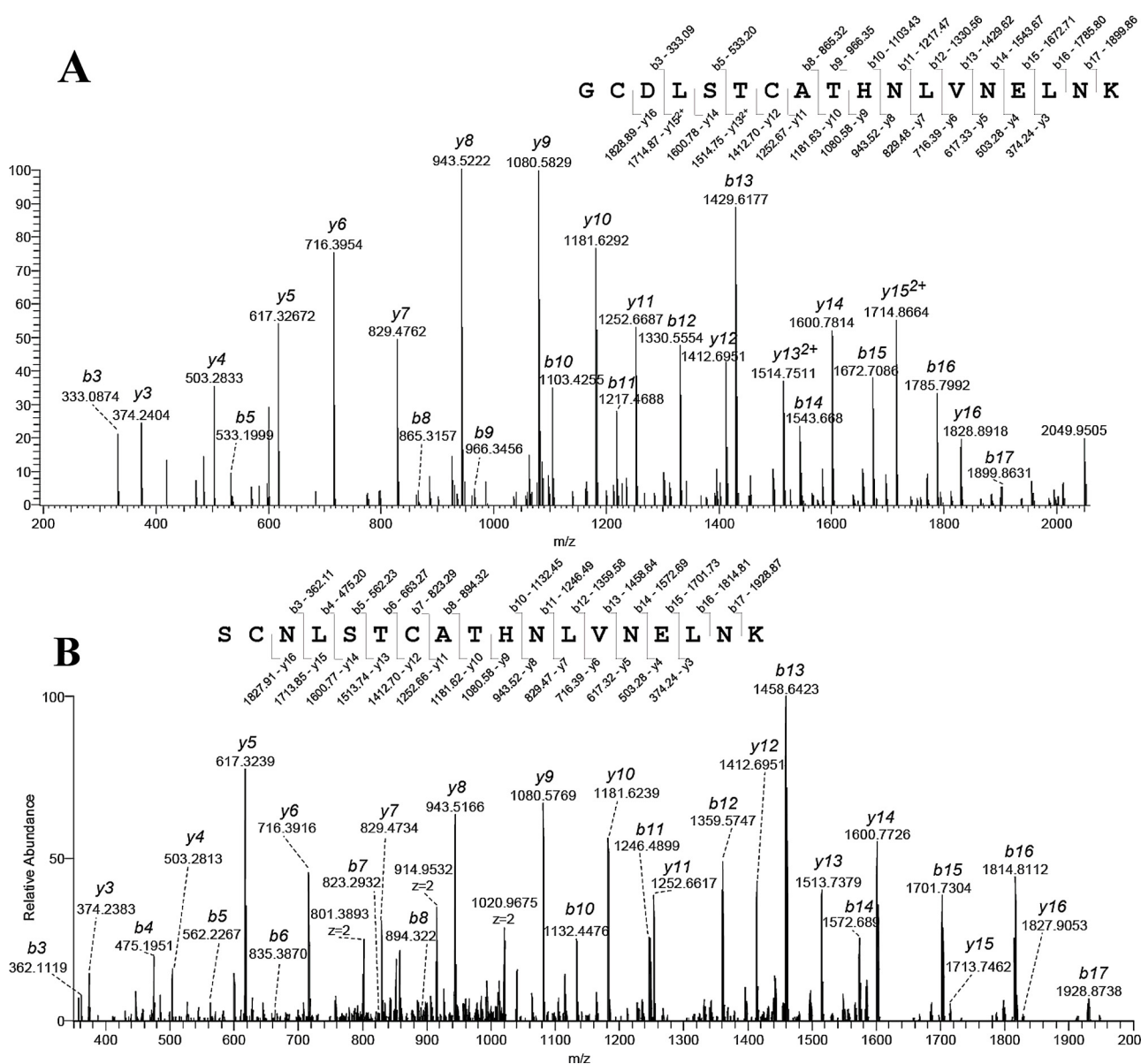


Fig. 4. Deconvoluted CID spectra of N-terminal tryptic fragment of OsCTLP-1 (A) and -2 (B), containing IAM alkylated cysteines. Selected precursor m/z values of doubly charged ions were 1037.97 and 1111.52, respectively.

and light labeled peptides were the same until y_{12} (Lys_2), where a shift in mass of 4Da was observed in the heavy labeled peptide (Fig. 7) due to incorporation of deuterated dimethyl. The first b-ions sequenced on heavy and light labeled peptide, however, included the Lys besides the aminoterminal residue, showing, therefore, a shift of 8 Da in all b-ion peaks. This labeling experiment combined with high mass accuracy orbitrap analysis affirmed the assignment of the C-terminal sequence as SKPSSGGVGPESF.

Ultimate onfirmation of the primary structure of OsCTLP-2, especially residues 1 and 2, and also differentiation between Leu and Ile residues were achieved by Edman degradation.

Finally the high sequence homology between OsCTLP-1 and -2 prompts us to speculate that the final sequence of OsCTLP-1 also has all Leu rather than Ile residues: GCDLSTCATHNLVNEELNFKDKSPSSGGVGPESF-NH₂.

NCBI BLAST analysis of OsCTLP-1 and -2 reveals more than 50% identity with adrenomedullin (ADM)-like peptides from *Ornithodoros* ticks. BLAST with anura restriction yielded 27 to 40% identity with calcitonin (CT) and calcitonin gene related peptide (CGRP)

from *Rana catesbeiana*, *Rana ridibunda* and *Phyllomedusa bicolor*. Comparisons of OsCTLP-1 and -2 with other (mainly) vertebrate CT, CGRP and ADM peptides are listed in Tables 1–3.

4. Discussion

We elucidated the primary structures of two related peptides from the skin secretion of the Chinese odorous frog *O. schmackeri*, which are both characterized by a single intramolecular disulfide bridge. Both peptides could be fully *de novo* sequenced employing a combination of different analytical tools. The successful strategy included comparative CID and ETD tandem MS fragmentation analyses of the native peptides and of their reduced forms alkylated with two different alkylation chemicals. In addition high resolution MS/MS analysis after trypsin digestion, partial carboxypeptidase Y treatment, dimethyl labeling, and finally also Edman degradation contributed to the full sequence assignment.

CID and ETD spectra are known to be complementary particularly for *de novo* sequencing of larger peptides [16], and

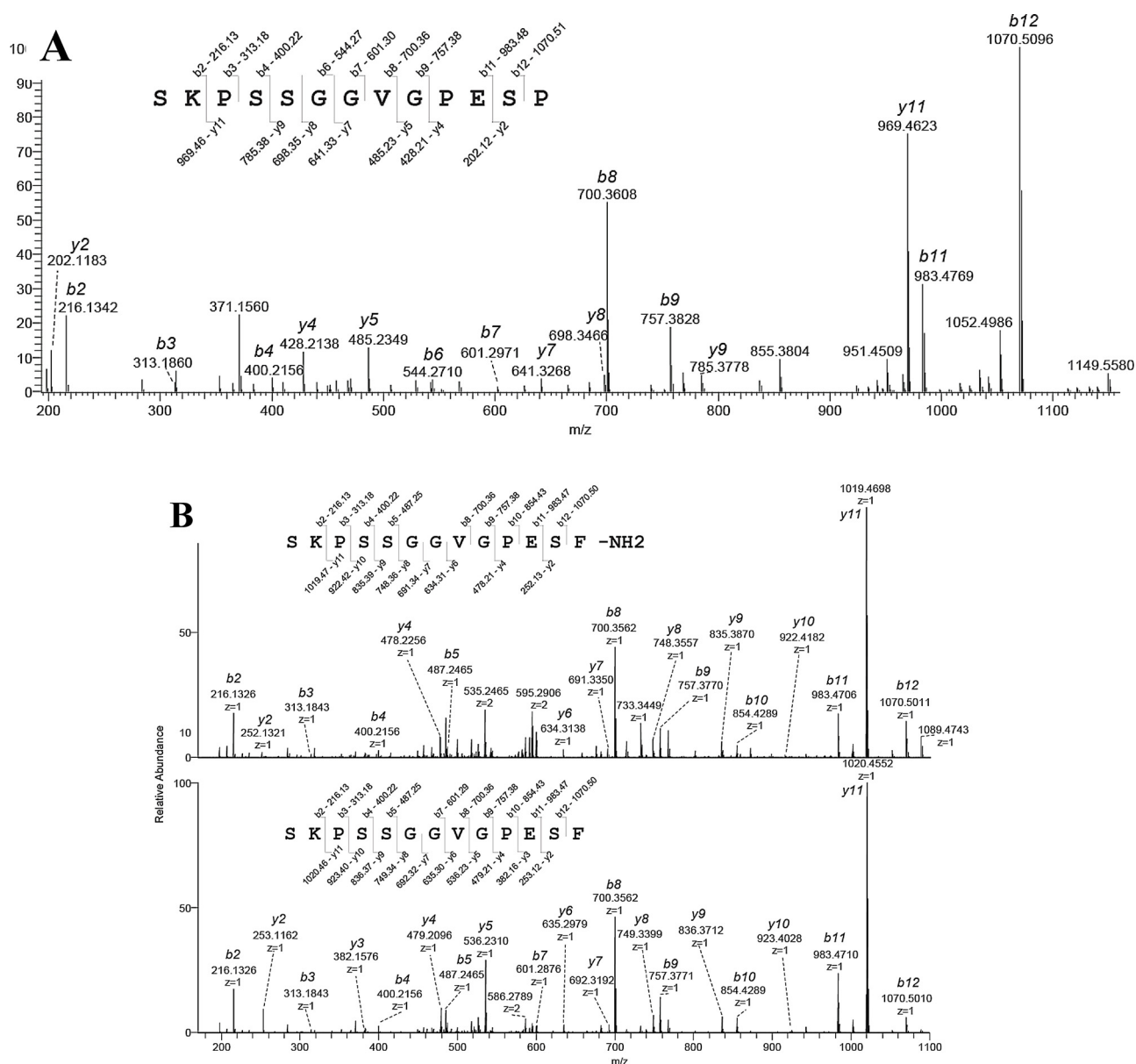


Fig. 5. Deconvoluted CID spectra of C-terminal tryptic fragment of OsCTLP-1 (A) and OsCTLP-2 (B). B shows both amidated (top; m/z [617.8037]²⁺), and non-amidated [618.2974]²⁺ variant.

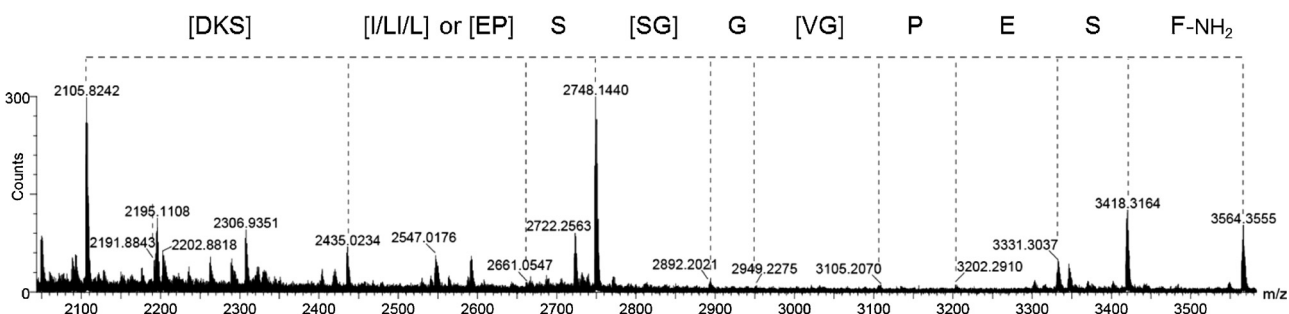


Fig. 6. MALDI-TOF of fraction 16 containing OsCTLP-2 after 100 min. incubation with CPY. C-terminal sequence can be read until 10th residue. Digestion after 2661 Da was no longer informative.

this is nicely illustrated for both peptides sequenced in this paper (Figs. 2–3). Cysteine alkylation with BrEA in particular, was found to promote higher charge state peptide precursors compared to

IAM treatment (illustrated in Fig. 1E and F), which is beneficial for improved ETD fragmentation [17–20]. Higher intensity of b-/y-ions (CID) aminoterminally from Pro-residues, and the absence of this

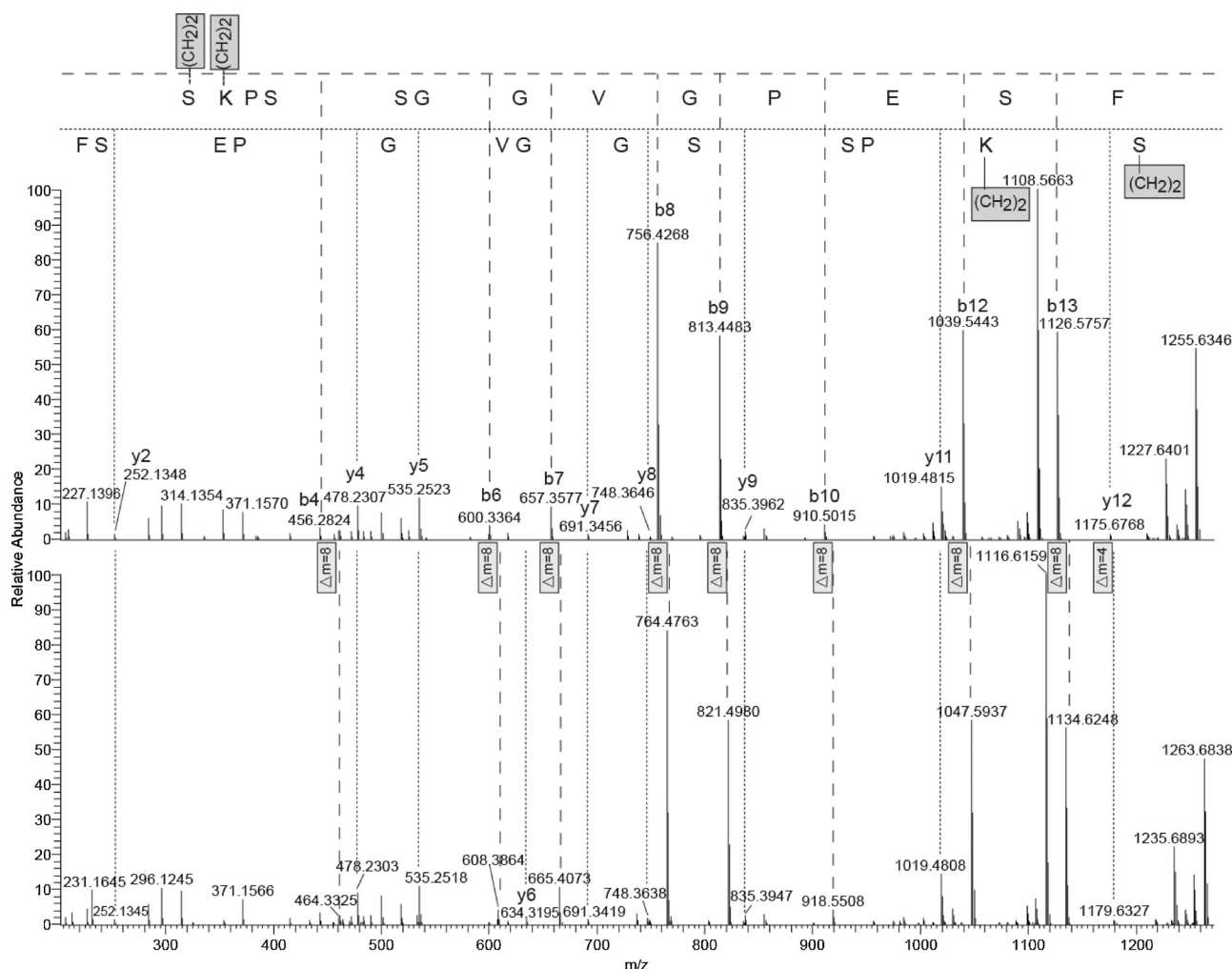


Fig. 7. Deconvoluted CID spectra of OsCTLP-2C-terminal tryptic fragment labeled with light ([645.8420 + 2H]²⁺, top) and heavy ([649.8661 + 2D]²⁺ bottom) dimethyl. Both Ser¹ and Lys² are labeled, as evident from mass increase of b4 ion of 56 Da (top, light label), and 64 Da (bottom, deuterated label). Hence difference of 8 Da observed in all further b-ion peaks. Ions of y-series yield identical values for heavy and light labeled peptide until y11. Ion y12 carries a dimethylated Lys, with concomitant mass increase to 156.13 Da (light version) and 160.15 Da (heavy version). Non-annotated y3 (381.1776) and y10 (922.4301 and 922.4270) ions were present in native spectra (non-deconvoluted) and helped to confirm peptide sequence.

Table 1
Vertebrate calcitonin (CT) sequence homologies with OsCTLP-1 and -2.

Species	Calcitonin sequences	Similarity with OsCTLP-1 (%) and -2 (%)	
<i>O. schmackeri</i> CTLP-1	GC-DLSTCATHNLVNLNKFDSKPPSSGGVGPESP	100	91
<i>O. schmackeri</i> CTLP-2	SC-NLSTCATHNLVNLNKFDSKPPSSGGVGPESF	91	100
<i>Sardinops melanostictus</i>	CSNLSTCALGKLSQELHKLQS-YPRTN-VGAGTP	43	41
<i>Sus scrofa</i>	CSNLSTCVLSAYWRNLNNFHR-FSGMG-FGPETP	40	38
<i>Rana catesbeiana</i>	CSGLSTCALMKLSQDLHRFNS-YPRTN-VGAGTP	40	35
<i>Bos taurus</i>	CSNLSTCVLSAYWKDLNNYHR-FSGMG-FGPETP	37	35
<i>Canis lupus familiaris</i>	CSNLSTCVLGTYSKDLNNFHT-FSGIG-FGAETP	37	35
<i>Carassius auratus</i>	CSSLSTCVLGKLSQELHKLQT-YPRTN-VGAGTP	37	31
<i>Gallus gallus</i>	CASLSTCVLGKLSQELHKLQT-YPRTD-VGAGTP	37	29
<i>Oryctolagus cuniculus</i>	CGNLSTCMLGTYTQDLNKFHT-FPQTA-IGVVAP	34	36
<i>Homo sapiens</i>	CGNLSTCMLGTYTQDFNKFHT-FPQTA-IGVGAP	34	34

cleavage in ETD were additional mass spectral features that assisted with sequence assignment (Figs. 2–5, 7).

The identification of 2 Lys-residues in the middle of the long sequence, prompted us to attempt additional CID fragmentations on the smaller tryptic peptides. Ambiguities at the C-terminal end (the exact order of the 3rd and 4th C-terminal residues: PE rather than EP) were resolved by exoprotease carboxypeptidase Y treatment. High resolution MS helped to accurately calculate the

correct sequence possibility to fill ‘gaps’ of 2 and 3 residues in incomplete b/y-ion series. Labeling with heavy and light dimethyl also yielded informative data. By comparing heavy and light labeled spectra, y and b-ions were easily distinguished and consequently assigned (Fig. 7).

Sequence homology analysis (BLAST) of the OsCTLP primary structures reveals shared conserved motifs with calcitonin (CT), calcitonin gene-related peptide (CGRP), and adrenomedullin

Table 2

Calcitonin gene-related peptide (CGRP) sequence homologies with OsCTLP-1 and -2.

Species	CGRP sequences	Similarity with OsCTLP-1 (%) and -2 (%)	
<i>O. schmackeri</i> CTLP-1	GCDLSTCATHNLVNLNKF-DKSKPSSGGVGPESP	100	91
<i>O. schmackeri</i> CTLP-2	SCNLSTCATHNLVNLNKF-DKSKPSSGGVGPESF	91	100
<i>Ornithodoros coriaceus</i>	TVCNAATCATGNLAAQLSGGG-KSKSPANSTGTEGF	37	43
<i>Oryctolagus cuniculus</i>	GCNTATCVTHRLAGLLSRSGGMVKSNEFVPTNVGSEAF	35	38
<i>Phyllomedusa bicolor</i>	SCDTSTCATQRLADFLSRSGGIGSPDFVPTDVSANSF	32	35
<i>Homo sapiens</i>	ACNTATCVTHRLAGLLSRSGGMVKSNEFVPTNVGSKAF	30	35
<i>Homo sapiens</i>	ACDTATCVTHRLAGLLSRSGGVVKNNEFVPTNVGSKAF	30	30
<i>Rana ridibunda</i>	ACNTATCVTHRLADFLSRSGGMVKSNEFVPTNVGSKAF	27	32
<i>Gallus gallus</i>	ACNTATCVTHRLADFLSRSGGVKNNEFVPTNVGSKAF	27	32

Table 3

Adrenomedullin (ADM) sequence homologies with OsCTLP-1 and -2.

Species	Adrenomedullin sequences	Similarity with OsCTLP-1 (%) and -2 (%)	
<i>O. schmackeri</i> CTLP-1	GCDLSTCATHNLVNLNKF-DKSKPSSG-GVGPESP	100	91
<i>O. schmackeri</i> CTLP-2	SCNLSTCATHNLVNLNKF-DKSKPSSG-GVGPESF	91	100
<i>Ornithodoros parkeri</i>	GCSLSTCVLQKLSKLNHFTDDSKNKG-GTGPDYS	54	51
<i>O. cuniculus</i>	YRQSMKNFQGSRSFGCRFGTCTVQNLAHQIYQFTDKDKDDTAPRNKISPQGY	21	19
<i>H. sapiens</i>	YRQSMNFGQLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY	19	17

Bioassays on rat smooth muscle preparations containing bradykinin B1 and B2-type receptors did not reveal any myotropic activity of either of the two synthetic peptides. Up to micromolar concentration none of the peptides elicited marked effects on the contraction of rat ileum, bladder, uterus or tail artery. The same preparations did show the expected response to bradykinin as a positive control.

(ADM), from different animal species (Tables 1–3). Both novel peptides share an aminoterminal Cys_{2–7} disulfide bridge with CGRP. It is interesting to note that OsCTLP-2 also shares its carboxyterminal end (a carboxyamidated Phe) with CGRP, whereas this residue is an amidated Pro in OsCTLP-1, which is a feature typical of all CTs.

All these features make that the BLAST algorithm classifies the novel peptides as members of the calcitonin gene-related peptide superfamily, which prompted us to call the peptides OsCTLPs. The calcitonin gene-related peptide superfamily comprises CTs, CGRPs, and ADMs (as well as the mammalian calcitonin receptor-stimulating peptides (CRSP), and amylin (AMY)). CTs and CGRPs typically have 32 and 37 residues each and are characterized by a conserved intramolecular disulfide bridge (between Cys_{1–7} in CT and Cys_{2–7} in CGRP), a carboxyterminal amide and a consensus primary structure, particularly at the N-/C-termini [21,22]. Their sequence homology originate from their related genes. In vertebrates, CT and CGRP are known to be both products of the same calcitonin (CALC) gene, which is expressed in specific tissues by alternative exon splicing into specific mRNAs. In the amphibian ultimobranchial glands and in the mammalian thyroid gland the mature mRNA is transcribed from the CT exon [23], whereas in the vertebrate central and peripheral nervous system, the expressed mRNA consists of the CGRP exon [24,25].

Within this family, the frog CGRPs as identified in *Rana catesbiana* (from ultimobranchial gland [26]), *Rana ridibunda* (brain and intestine [21]) and in *Phyllomedusa bicolor* [27] show considerable sequence variety. It is intriguing that the OsCTLPs occur in the skin secretion of *O. schmackeri*. So far only in *Phyllomedusa bicolor*, a CGRP has been identified in the skin. Its sequence is identical to that of the brain and intestine [27]. CGRP, therefore, fits in the brain-gut-skin triangle concept of Erspamer, that every peptide found in the skin secretion of frogs has its counterpart in the brain and gut of mammals [6,7].

The *O. schmackeri* peptides described here, however, are not typical CGRPs (see Table 2). The homology is less than between typical vertebrate CGRPs, and they are 3 residues shorter (34 i.s.o. 37). For similar reasons the OCTLPs do not qualify as 'true' CTs (see Table 1), which are typically 2 residues shorter.

The third family of peptides the BLAST algorithm identifies as homologous to OsCTLPs include those originating from the ADM gene. These peptides are typically considerably longer (52 residues), but have critical residues at 'conserved' positions, such as Cys₂, Cys₇, Phe₁₉, three Lys and a Pro at position 4 from the amidated C-terminus. In this respect it is interesting to note that ADM immunoreactivity has been detected in the skin of *Xenopus laevis* (similar to the situation in human skin) [28].

About the function of the novel peptides in the skin of *O. schmackeri*, we can only speculate. Biological effects of members of the calcitonin gene related peptide superfamily are plentiful. CT typically plays a role in bone calcification. It lowers blood calcium levels [29] by stimulating Ca²⁺ absorption by the intestine and renal calcium excretion [30,31]. CGRP exhibits a wide array of bioactivities including pain perception (high blood levels are correlated with migraine, arthritis, temporomandibular-joint disorders and many postmenopausal symptoms and diseases [32–34]), neurogenic inflammation. ADM bioactivity includes antimicrobial activity, renal homeostasis, hormone regulation, neurotransmission, growth modulation and vasodilation. As also CGRPs have dramatic potent effects on vasodilation and heart beat regulation [32,33,35], and as vasodilatory peptides comprise part of the defense strategy of frogs against predators (e.g. bradykinins, bradykinin potentiating peptides [15] we considered it worthwhile testing the OCTLPs on mammalian (rat) smooth muscle preparations. However the tissues we selected did not prove suitable to demonstrate any myotropic activity by the novel OCTLPs. To determine the pharmacology of these peptides, additional alternative bioassays are required. These may include preparations of muscles of non-mammalian origin, but also various cellular tests assaying, e.g. for bone calcification, as well as for antimicrobial activity.

Follow-up research will thus be necessary to establish the role of OCTLPs in frog skin biology. To more extensively study the genetic relationship between OCTLPs and CTs, CGRPs and ADMs, the nucleic acid sequences from an *O. schmackeri* skin secretion will be analyzed from a cDNA library constructed from reverse transcribed poly-adenylated mRNAs, using primers derived from the sequences established.

5. Conclusion

We report a tandem MS based analytical strategy to fully *de novo* sequence two >30 amino acid residue peptides directly from LC separation of skin secretion of the Chinese frog, *O. schmackeri*. Because of the sequence homology with peptides of the calcitonin/CGRP superfamily of peptides, we designated the peptides OsCTLP-1 and OsCTLP-2, the number reflecting their elution order in reverse phase HPLC.

Of OsCTLP-2, a sufficient amount could be purified for automated Edman degradation. The full Edman analysis required prior tryptic cleavage of the pure peptide in two halves: the N-terminal octadecapeptide, and the C-terminal hexadecapeptide. Both peptide halves were in turn purified by HPLC after which their primary structure could be fully called until their carboxyterminal residue by Edman sequencing. The latter analysis unequivocally solved the I/L ambiguities which remained after MS analysis at positions 4, 12 and 16 of the N-terminal half of the OsCTLP-2.

In view of the high sequence homology between both peptides, it is likely that also in OsCTLP-1, residues 4, 12 and 16 are all L.

We, thence, report two novel peptide sequences, which may be prototypes of a new family within the calcitonin/CGRP superfamily of peptides:

OsCTLP-1: GCDLSTCATHNLVNLNKFDSKPSSGGVGPESP-NH₂
and OsCTLP-2: SCNLSTCATHNLVNLNKFDSKPSSGGVGPESF-NH₂.

Peptide sequence deposition details

The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession numbers COHJV0 and COHJV1 for OsCTLP-1 and OsCTLP-2 respectively.

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